M-PTO-1390 (Rev. 12-29-99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 003300-816

U.S. APPLICATION NO. (If known, see 37 C F.R. 1.5): Unassigned 09/890949

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/SE00/00210	3 February 2000	9 February 1999

TITLE OF INVENTION: MONOCLONAL ANTIBODY

APPLICANT(S) FOR DO/EO/US: JOHAN STENFLO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (Signed Declaration will follow)
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

A certified copy of Swedish Application No. 9900431-9, filed 9 February 1999, was submitted during the international phase of the examination. Thus, the claim for priority has been perfected.

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unassigned 09/890949	PCT/SE00/00210	003300-816

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00 (960)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00 (970)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00 (958)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00 (956)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1,000.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	24 -20 =	4	X$18.00 (966)	$ 72.00	
Independent Claims	1 -3 =	0	X$80.00 (964)	$ ---	
Multiple dependent claim(s) (if applicable)			+$270.00 (968)	$ ---	

TOTAL OF ABOVE CALCULATIONS =	$ 1,072.00	
Reduction for 1/2 for filing by small entity, if applicable (see below).	$ ---	-
SUBTOTAL =	$ 1,072.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +	$ ---	
TOTAL NATIONAL FEE =	$ 1,072.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +	$ ---	
TOTAL FEES ENCLOSED =	$ 1,072.00	
	Amount to be: refunded	$
	charged	$

a. ☐ Small entity status is hereby claimed.

b. ☒ A check in the amount of $ 1,072.00 to cover the above fees is enclosed.

c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Filed: August 8, 2001

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Benton S. Duffett, Jr.
NAME

22,030
REGISTRATION NUMBER

Patent
Attorney's Docket No. 003300-816

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	**BOX PCT**
JOHAN STENFLO	)	
	)	**Attn: DO/EO/US**
Application No.: (unassigned)	)	
	)	Group Art Unit: (Unassigned)
Filed: August 8, 2001	)	
	)	Examiner: (Unassigned)
For: MONOCLONAL ANTIBODY	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a national phase filing of International Application No. PCT/SE00/00210, filed February 3, 2000.

Please amend the above-identified Application as indicated.

IN THE ABSTRACT:

Please add the Abstract of the Disclosure that is provided on a separate sheet.

IN THE CLAIMS:

Kindly replace Claims 4 to 6, 8, 15, 16 and 19 as indicated below.

4. (Amended) A monoclonal antibody according to claim 1, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator

(uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGKI and prostatic specific antigen (PSA).

5. (Amended) A monoclonal antibody according to claim 1, wherein said inhibitor is protein C inhibitor (PCI) or α_1-antitrypsin.

6. (Amended) A method for preparation of a monoclonal antibody as defined in claim 1, wherein an animal is immunised with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved form of said inhibitor, followed by screening for and isolation of said monoclonal antibody.

8. (Amended) A method for monitoring the activity of systems involving protein C inhibitor, wherein a monoclonal antibody as defined in claim 1 is used in an immunoassay.

15. (Amended) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monoclonal antibody according to claim 1 is utilised.

16. (Amended) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to claim 8 is utilised.

19. (Amended) A kit for qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody according to claim 1.

Please cancel Claims 17 and 18 without prejudice.

Please add the following new Claims 20 to 26.

20. (New) A monoclonal antibody according to claim 2, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGKI and prostatic specific antigen (PSA).

21. (New) A monoclonal antibody according to claim 2, wherein said inhibitor is protein C inhibitor (PCI) or α_1-antitrypsin.

22. (New) A method for preparation of a monoclonal antibody as defined in claim 2, wherein an animal is immunised with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved form of said inhibitor, followed by screening for and isolation of said monoclonal antibody.

23. (New) A method for monitoring the activity of systems involving protein C inhibitor, wherein a monoclonal antibody as defined in claim 2 is used in an immunoassay.

24. (New) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monoclonal antibody according to claim 2 is utilised.

25. (New) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to claim 2 is utilised.

26. (New) A kit for qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody according to claim 2.

REMARKS

The present amendment adds an Abstract of the Disclosure on a separate sheet and modifies the form only of the claims while eliminating use Claims 17 and 18 and discontinuing multiple dependency.

The examination and allowance of the Application are respectfully requested.

Respectfully submitted,

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Date: August 8, 2001

Attachment to Preliminary Amendment dated August 8, 2001

Abstract of the Disclosure

The present invention relates to a monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, a method for preparation of said monoclonal antibody, a method for monitoring the activity of systems involving protein C inhibitor and a method for diagnosis of e.g., venous thrombosis, wherein said monoclonal antibody is utilised. Said monoclonal antibody is suitable for monitoring the activity of systems involving protein C inhibitor, and it has specific affinity for both i) a complex between a serine proteinase and an inhibitor thereof, and ii) a cleaved and uncomplexed form of said inhibitor, but has substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or a derivative thereof having the same biological activity.

Attachment to Preliminary Amendment dated August 8, 2001

Marked-up Claims 4 to 6, 8, 15, 16 and 19

4. (Amended) A monoclonal antibody according to [any one of the preceding claims] claim 1, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGKI and prostatic specific antigen (PSA).

5. (Amended) A monoclonal antibody according to [any one of the preceding claims] claim 1, wherein said inhibitor is protein C inhibitor (PCI) or α_1-antitrypsin.

6. (Amended) A method for preparation of a monoclonal antibody as defined in [any one of claims 1-5] claim 1, wherein an animal is immunised with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved form of said inhibitor, followed by screening for and isolation of said monoclonal antibody.

8. (Amended) A method for monitoring the activity of systems involving protein C inhibitor, wherein a monoclonal antibody as defined in [any one of claims 1-5] claim 1 is used in an immunoassay.

Attachment to Preliminary Amendment dated August 8, 2001

Marked-up Claims 4 to 6, 8, 15, 16 and 19

15. (Amended) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monoclonal antibody according to [any one of claims 1-5] claim 1 is utilised.

16. (Amended) A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to [any one of claims 8-14] claim 8 is utilised.

19. (Amended) A kit for qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody according to [any one of claims 1-5] claim 1.

MONOCLONAL ANTIBODY

Field of the Invention

The present invention relates to a monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, a method for preparation of said monoclonal antibody, a method for monitoring the activity of systems involving protein C inhibitor and a method for diagnosis of *e.g.* venous thrombosis, wherein said monoclonal antibody is utilised.

Background of the Invention

Protein C is a proenzyme to "activated protein C". Activated protein C, hereinafter denoted APC, is a serine proteinase, and its proenzyme protein C is a liversynthesised glycoprotein having 461 amino acid residues and a molecular weight of approximately 62 kDa. Protein C carries two parts denoted the light and the heavy chain, respectively (formed by limited proteolysis of a single chain precursor). The light chain is glycosylated on its Asn 97 residue, whereas the heavy chain is glycosylated on its Asn 248, Asn 313 and Asn 329 residues (Asn 329 being glycosylated only in so-called "single chain protein C"). Of all the protein C in plasma, about 85% contains both the light and heavy chain, whereas about 15% consists of single chain protein C. There is no known difference in biological activity between these two forms of protein C.

The light chain of protein C contains an amino-terminal domain with nine γ-carboxyglutamic acid (Gla) residues, which have been formed by vitamin K-dependent carboxylations. This domain is followed by two domains, both of which are homologous to the epidermal growth factor (EGF) precursor. Of said two domains, one has a single hydroxylated aspartic acid residue, *i.e.* consists of *erythro*-β-hydroxyaspartic acid. The light and the heavy chains are linked together via one disulfide bond only.

Moreover, protein C requires vitamin K for its normal biosynthesis, and it circulates in blood plasma at a concentration of approximately 4 mg/l.

Protein C is an important regulator of blood coagulation, and it is activated by thrombin complexed with thrombomodulin. This activation involves removal of an activation peptide with 12 amino acids from the amino-terminal end of the heavy chain of protein C, whereby APC is formed. APC is a typical serine proteinase with high arginyl bond specificity.

The substrates degraded by APC are the cofactors known as factor V/V_a and factor VIII/$VIII_a$, respectively, where "$_a$" denotes the active form of the cofactor. In coagulation pathways, the factor $VIII_a$ forms a membrane-bound complex with the factor IX_a, whereby the coagulation factor X is activated to a serine proteinase denoted coagulation factor X_a. Furthermore, the factor V_a forms a membrane-bound complex with the coagulation factor X_a, whereby prothrombin is activated to thrombin.

The degradation of the factors V_a and $VIII_a$ by APC requires two cofactors. These are protein S, which is a vitamin K-dependent protein, and factor V, which is the unactivated form of factor V_a. The degradation results in cleavage of the factors V_a and $VIII_a$, whereby their affinity for factors X_a and IX_a, respectively, is reduced. Thus, said cleavage results in a reduction of the rate of formation of both thrombin and coagulation factor X_a. In other words, the blood coagulation cascade is turned off.

As other serine proteinases, APC has its inhibitors. These are protein C inhibitor (hereinafter denoted PCI; plasma concentration approximately 4 mg/l), α_1-proteinase inhibitor (hereinafter denoted α_1-antitrypsin; plasma concentration approximately 1 g/l) and α_2-macroglobulin (plasma concentration approximately 2 g/l). PCI and α_1-antitrypsin belong to a group of inhibitors sometimes referred to as serpines (serine proteinase inhibitors), and form 1:1 complexes with APC.

The complex formation between APC and PCI proceeds at a slow rate, and it proceeds even slower in respect of α_1-antitrypsin. This slow rate is manifested by a long half-life for APC in plasma, wherein $t_{1/2}$ is about 20 min. However, the rate of complex formation between APC and PCI is increased by heparin.

Upon complex formation with APC, the serpin is cleaved in its so-called bait region, whereby a stable intermediate acyl complex is formed. With time, the intermediate acyl complex dissociates, whereby APC is regenerated and a proteolytically modified, *i.e.* cleaved, inactive serine proteinase inhibitor is formed.

The intermediate acyl complexes are removed rather rapidly from the circulatory system. $T_{1/2}$ in baboons is approximately 40 and 140 min for the APC:PCI and APC:α_1-antitrypsin complexes, respectively. PCI appears to be the prime inhibitor for APC with α_1-antitrypsin and α_2-macroglobulin taking over as the PCI concentration is substantially lowered.

PCI also inhibits thrombin and factor X_a, both of which are blood coagulation proteinases. In addition, PCI inhibits trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, prostatic specific antigen (PSA) and the prostata specific kallikrein-like serine proteinase denoted HGK1.

The biological importance of APC can be illustrated in several ways, *e.g.*

i) newborn children with homozygous protein C deficiency die during the first days of life in a clinical condition denoted *purpura fulminans*, which is the result of widespread thrombotisation of capillaries;

ii) heterozygosity for protein C deficiency has in many cases been associated with a thrombofilia that accounts for approximately 2% of all hereditary thrombofilia;

iii) a frequently occurring (present in 2-10% of caucasian populations) point mutation in factor V, rendering factor V/V_a resistant to degradation by APC, is associated with a mild form of hereditary thrombofilia in the heterozygous form and a more severe form of thrombofilia in the homozygous form;

iv) disseminated intravascular coagulation is associated with a depletion of activated protein C, a process which often leads to a fatal outcome;

v) gram negative septicemial (caused by *e.g.* meningococci) leads to a depletion of activated protein C, a condition shown to be fatal in baboons as well as in humans.

In summary, the considerable biological importance of serine proteinases, particularly protein C, as regulators of blood coagulation as well as their role as key proteins in the so-called protein C anticoagulant pathway has resulted in a great need in the art to develop powerful research tools and extract clinically relevant information from *e.g.* the concentration of APC in plasma.

In particular, such clinically relevant information could be very useful in the diagnosis of *e.g.* venous or arterial thrombosis, including coronary infarction or pulmonary embolism.

Prior Art

To serve the above-mentioned purposes and measure the activation of anticoagulant systems involving protein C, numerous methods have been developed, as set forth below.

In Bauer, K.A., Kass, B.L., Beeler, D.L., Rosenberg, R.D., *J. Clin. Invest.*, **74**:2033-2041 (1984), a method is disclosed, wherein the plasma concentration of the activation peptide removed from protein C (*vide supra*) is measured. Since said activation peptide has a very high renal clearance, its plasma concentration is low. Thus,

this method is cumbersome as it requires large amounts of plasma.

In Gruber, A., Griffin, J.H., *Blood*, **79**:2340-2348 (1992), a method is disclosed, wherein the plasma concentration of APC is measured. Here, an ELISA technique with a catching monoclonal antibody against protein C was used. Since said catching monoclonal antibody does not inhibit the activity of APC, an amidolytic assay was used. However, due to the low plasma concentration of APC, the incubation time was generally very long (up to days) and impractical.

In an approach disclosed in Espana, F., Zuazu, I., Vicente, V., Estelles, A., Marco, P., Aznar, J., *Thromb. Haemost.*, **75**:56-61 (1996), heparin-mediated enhancement of the rate of complex formation between APC and PCI is utilised. This method requires the presence of *e.g.* heparin, benzamidine and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPAC).

However, the most relevant approaches disclosed relate to methods, wherein the concentration of a complex between APC and PCI, or between APC and α_1-antitrypsin, is measured by use of monoclonal antibodies. These approaches are all based on the assumption that said concentration reflects the concentration of APC, and hence also the degree of activation of the protein C anticoagulant system. Here, a catching monoclonal antibody raised against either APC or PCI is used together with a suitable tracer agent in an ELISA technique.

Thus, if the catching monoclonal antibody is specific for PCI, the tracer agent could be an antibody specific for an epitope in protein C, or vice versa. However, an inherent problem in this approach is that the concentration of free, uncomplexed PCI and APC, respectively, is very high in comparison with the APC:PCI complex to be measured. As an example, due to competitive binding between free, uncomplexed PCI and complex-bound PCI to a catching monoclonal antibody raised against PCI, the

sensitivity of such an approach is greatly reduced. Typical such approaches are disclosed in JP 02-236 452, Espana, F., Griffin, J.H., *Thromb. Res.*, **55**:671-682 (1989) and Espana, F., Vicente, V., Scharrer, I., Tabernero, D., Griffin, J.H., *Thromb. Res.*, **59**:593-608 (1990).

In an approach disclosed in Minamikawa, K., Wada, H., Wakita, Y., Ohiwa, M., Tanigawa, M., Deguchi, K., Hiraoka, N., Huzioka, H., Nishioka, J., Hayashi, T., *Thromb. Haemost.*, **71**:192-194 (1994), a precipitated barium salt is used to bind PCI in complex with APC, whereas uncomplexed PCI does not bind to the precipitated barium salt. After centrifugation and dissolution in EDTA (aq), the sample is subjected to ELISA, wherein a catching monoclonal antibody raised against PCI is used. Although useful from a sensitivity point of view, this approach is time-consuming as well as laborious.

In Laurell, L., Carlson, T.H., Stenflo, J., *Thromb. Haemost.*, **60**:334-339 (1988), it is disclosed monoclonal antibodies raised against cleaved PCI, wherein said monoclonal antibodies are applied in an assay for measuring the plasma concentration of APC:PCI complexes. All of said monoclonal antibodies also had specific affinity for uncleaved, uncomplexed PCI.

In WO 9822509, there is disclosed monoclonal antibodies which bind specifically to the PSA-ACT complex without significant cross-reactivity with PSA, ACT or CG-ACT. These antibodies are not suitable for monitoring the activity of systems involving protein C inhibitor.

In summary, there is still a great need in the art for a monoclonal antibody with high specific affinity for a serine proteinase inhibitor, *e.g.* PCI, in complex with a serine proteinase, *e.g.* APC, where said monoclonal antibody has substantially no affinity for PCI in its uncomplexed form.

Disclosure of the Invention

According to the invention, there is now provided a new monoclonal antibody which overcomes the problems in the art referred to above. The monoclonal antibody according to the invention is suitable for monitoring the activity of systems involving protein C inhibitor, such as anticoagulant systems, and said monoclonal antibody is characterised by having specific affinity for both

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said inhibitor,

while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or being a derivative thereof having the same biological activity.

The monoclonal antibody according to the present invention is obtainable by immunisation of an animal with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody. Said animal is preferably a mouse, most preferably a Balb/c mouse.

Preferably, said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

As said inhibitor, either protein C inhibitor (PCI) or α_1-antitrypsin is preferred.

In the most preferred embodiment, the monoclonal antibody according to the invention is obtainable, or obtained, by immunisation with a *mixture* of cleaved PCI and PCI in complex with PSA.

In another aspect, the present invention also relates to a method for preparation of a monoclonal antibody as defined above, wherein an animal is immunised with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody.

In yet another aspect, the present invention relates to a method for monitoring the activity of anticoagulant systems involving protein C, wherein a monoclonal antibody as defined above is used in an immunoassay.

Preferably, said immunoassay comprises a sandwich-type immunoassay. More preferably, said sandwich-type immunoassay is a DELPHIA®, ELISA or magnetic bead technique comprising a tracer agent and said monoclonal antibody bound to a surface.

In an embodiment of said method, said tracer agent comprises an antibody having specific affinity for said serine proteinase or an epitope shared by said serine proteinase and said inhibitor.

In another embodiment, said tracer agent is conjugated to a suitable enzyme and/or labelled with a tracing substance. Said enzyme is *e.g.* an alkaline phosphatase, horse radish peroxidase or a β-galactosidase. Said tracing substance is *e.g.* ^{125}I, ^{131}I, Eu^{3+} or Sm^{3+} or a similar lanthanide.

In a further aspect, the present invention also relates to a method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monoclonal antibody as defined above is utilised.

Furthermore, this invention relates to a method for diagnosis of venous thrombosis, arterial thrombosis, em-

bolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a monitoring method as defined above is utilised.

Moreover, the present invention relates to the use of a monoclonal antibody as defined above for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

Also, this invention concerns the use of a monitoring method as defined above for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants In yet another aspect, the present invention relates to a kit for qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody as defined above.

Thus, the present invention allows for precise quantitative measurements of complexes between *e.g.* human APC and PCI in blood plasma. Indeed, the present invention encompasses measurements of complexes between PCI and thrombin or any other serine proteinase known to form complexes with PCI, *e.g.* coagulation factor X_a (this embodiment requires the exchange of the tracer agent only).

Some of the incentives for the use of a mixture of cleaved and complexed serine proteinase inhibitor for immunisation were gathered from mainly three sources. Firstly, it has been reported that certain monoclonal antibodies against the serpins antithrombin and C-1 esterase inhibitor (see *e.g.* de Agostini, A., Patston, P.A., Marottoli, V., Carrel, S., Harpel, P.C., Schapira, M., *J. Clin. Invest.*, **82**:700-705 (1988) and Asakura, S., Matsuda, M., Yoshida, N., Terukina, S., Kihara, H., *J. Biol. Chem.*, **264**:13736-13739 (1989)) are more or less selective for the complexed form of the inhibitor, albeit no such antibodies have been identified against PCI. Secondly, it has been disclosed in *e.g.* Björk, I.,

Nordling, K., Olson, S.T., *Biochemistry*, **32**:6501-6505 (1993) that thrombin:antithrombin complexes and antithrombin cleaved from such complexes share epitopes. These epitopes are not present on native antithrombin. Thirdly, it has been reported by *e.g.* Espana, F. and Griffin, J.H. (*vide supra*) that human seminal plasma has a PCI concentration that is about 50-fold higher in comparison with its blood plasma concentration. However, the PCI present in human seminal plasma is unable to form complexes with APC. Indeed, the PCI present in human seminal plasma is the cleaved form of PCI.

The monoclonal antibody according to the present invention, when used as catching antibody in *e.g.* a sandwich assay, has very high specific affinity for PCI in complex with APC as well as for the proteolytically cleaved PCI, but has substantially no or very low specific affinity for native PCI, *viz* PCI in its uncleaved and uncomplexed form. In other words, the native PCI has no influence on the binding of APC:PCI complexes to a surface having the monoclonal antibody according to the present invention bound thereto. Thus, there is substantially no competitive binding of the native PCI to said surface, whereby the sensitivity of the method according to the invention is high.

The method according to the present invention has been devised such that it can easily be automated and adopted for use in modern automated laboratory instrumentation using *e.g.* magnetic beads coated with the monoclonal antibody of the invention.

The present invention is applicable both as a powerful research tool and in conjunction with diagnosis of *e.g.* venous thrombosis, embolism and coronary infarction. In particular, the latter application could be very useful as it may detect coronary infarction much earlier than existing methods, most of which measure the concentration of enzymes or other proteins released from the damaged myocardium (CK-MB, troponin T or myoglobin). The

currently used methods are not diagnostically helpful until coronary ischemia has already damaged the myocardium. Detectable levels of CK-MB and troponin T are usually not present until 3-5 h (2-3 h for myoglobin) after the infarction, depending on its size.

Furthermore, the present invention may also help in the differentiation between coronary infarction and unstable angina, thereby improving current diagnostic procedures most significantly.

In a sandwich-type immunoassay, such as a DELPHIA® technique, two monoclonal antibodies are used. According to a preferred embodiment of the present invention, one has specific affinity for PCI, and this monoclonal antibody is bound to a surface and denoted "catcher" in a DELPHIA® context. The other monoclonal antibody has specific affinity against protein C and is denoted "tracer agent" in a DELPHIA® context. According to the present invention, the catcher has been carefully selected to circumvent the problems associated with the prior art (*vide supra*), *i.e.* that the large molar excess (normally more than 1000-fold) of uncomplexed protein, be it protein C or PCI, competes with the complexes to be measured by binding to a surface-bound monoclonal antibody.

Thus, in a preferred embodiment, the inventor has generated mouse monoclonal antibodies which have very high specific affinity for complexed PCI and cleaved PCI, but substantially no affinity for native PCI, *viz* PCI in its uncleaved and uncomplexed form. Such monoclonal antibodies are used as catcher in an immunoassay, as set forth below, and they were obtained by immunising Balb/c mice with a *mixture* of cleaved PCI and PCI in complex with a serine proteinase known as prostatic specific antigen (PSA). Furthermore, the inventor has devised approaches for carefully selecting, *i.e.* screening and isolating, such monoclonal antibodies, as is disclosed hereinbelow.

The present invention is further illustrated by the following non-limiting examples together with the accompanying figures.

Description of the figures

Fig. 1 shows affinity chromatograms for APC-complexed (A), cleaved (B) and native (C) PCI, respectively, obtained on an Affi Gel® 10 column (0,5 x 10 cm), onto which the monoclonal antibody M36 was immobilised. In each one of the samples A, B and C, 30 µg of the respective PCI analyte was chromatographed. The column was equilibrated with 50 mM Tris-HCl/0,5 M NaCl (pH 7,5). The flow rate was 0,1 ml/min. Bound protein was eluted with 0,1 M glycine-HCl/0,5 M NaCl with pH 2,7 (indicated by the arrow). The continuous line represents absorbance, whereas the "o-o"-line represents fluorescence. The early eluting peak in A consists of UV absorbing low molecular weight compounds and APC from cleaved complexes.

Fig. 2 shows measurements in real time of the interaction of APC:PCI complexes and cleaved PCI with the monoclonal antibody M36 using the surface plasmon resonance (SPR) technique. A) illustrates the interaction of APC:PCI complexes with M36, and the concentrations of the APC:PCI complexes were 17,6 nM (•), 8,8 nM (◆), 4,4 nM (▲) and 2,2 nM (▼). B) illustrates the interaction of cleaved PCI with M36, and the concentrations of cleaved PCI were 38,5 nM (•), 7,7 nM (▲), 3,9 nM (▼), 1,9 nM (■) and 1,0 nM (◆). The lines were fitted to the experimental data, some of which are denoted by the symbols.

Fig. 3 depicts dose-response curves for measurements of APC:PCI complexes. Each point represents the mean value of duplicate measurements. (◆) illustrates APC:PCI complexes in buffer, whereas (Δ) illustrates APC:PCI complexes in citrated plasma to which benzamidine has been added to a final concentration of 50 mM. The signal given by APC:PCI complexes prior to the addition of standard has been subtracted.

Examples

Preparation of proteins:

Native PCI was purified from human plasma according to a method described in Laurell, M. *et al.* (*vide supra*). Cleaved PCI and PCI in complex with PSA were purified from human seminal plasma using the same method.

Protein C was purified from human plasma by adsorption to barium citrate, elution with EDTA, column chromatography on DEAE Sepharose® and use of an immobilised monoclonal antibody against protein C (HPC-4) that recognises a calcium-dependent epitope in the N-terminal EGF-like domain, as disclosed in Ohlin, A.K., Stenflo, J., *J. Biol. Chem.*, **262**:13798-13804 (1987). Protein C was eluted from the column with 0,1 M sodium acetate/acetic acid buffer containing 0,5 M NaCl (pH 4,0). By addition of concentrated Tris buffer, an immediate adjustment to pH 7,5 was performed.

APC was prepared by thrombin-mediated activation of protein C, followed by isolation of APC by chromatography on an HPC-4 column, as described by Ohlin, A.K. and Stenflo, J. (*vide supra*).

Complexes used as standard in the method of measuring APC:PCI complexes were made by incubation of APC with a two-fold molar excess of PCI in 50 mM Tris-HCl, 0,1 M NaCl, 1 mM EDTA with pH 7,5. The complexes were isolated by affinity chromatography on an HPC-4 column, as described above. The purity of the isolated complex was established by SDS-PAGE and silver staining of the gel. The concentration of the complex in a standard solution was determined by measurement of Gla, assuming the presence of nine Gla residues per protein C molecule. Typically, the standard solution was made 0,1% in bovine serum albumin (BSA) and stored in aliquots at -70°C.

Used methods:

In the DELPHIA® assay, microtiter plates (Fluor Maxisorp, Nunc) coated with the monoclonal antibody M52 (1 µg/100 µl per well) and incubated overnight at +4°C

were washed with a wash solution (Wallac) and blocked with 0,7% BSA in an assay buffer (Wallac) for 2 h. After washing of the plates, 100 µl of each fraction, with a dilution of 1:1000 in assay buffer, was added to the wells. The plates were incubated for 1 h on a DELPHIA® plate shaker (Wallac) and then washed. Eu^{3+} labelled monoclonal antibodies M11-5 (B and C in figure 1) or HPC-4 (A in Fig. 1), having a dilution in assay buffer to a concentration of 20 ng/100 µl, were added, followed by 1 h incubation on the shaker. After washing, 200 µl enhancement solution (Wallac) was added, followed by 5 min incubation on the shaker. Fluorescence was then recorded in a DELPHIA® fluorimeter (Wallac).

Surface plasmon resonance (SPR) was measured with the BIAcore® technology (Pharmacia). Streptavidin was immobilised on the BIAcore® sensorchip CM5 according to the instructions of the manufacturer. The immobilised streptavidin gave between 5400 and 6800 response units (RU). Biotinylated antibody, having a concentration of 0,34 mg/ml in 10 mM Tris-HCl/150 mM NaCl (pH 7,75), was immobilised at a flow rate of 5 µl/min, which resulted in a response of 2800-3000 RU. Aliquots of protein stock solution were diluted in flow buffer and 20 µl was injected during the association phase at a constant flow rate of 5 µl/min. The dissociation phase was monitored at the same flow rate for 10 min. The system was regenerated by short pulses of 50 mM NaOH at a flow rate of 2 µl/min. For the fitting of the lines to the experimental data, see *e.g.* He, X., Shen, L., Malmborg, A.C., Smith, K.J., Dahlback, B., Linse, S., *Biochemistry*, **36**:3745-3754 (1997).

Production of monoclonal antibodies:

Balb/c mice were immunised with a mixture of PCI in complex with PSA (approximately 80%) and PCI cleaved from such a complex (approximately 20%). Each mouse was immunised three times with approximately 10 µg of said mixture each time. The two first immunisations were made in-

tracutaneously with said mixture emulsified in Freund's complete adjuvant, whereas the third immunisation was administered subcutaneously with Freund's incomplete adjuvant. Testing of the mouse plasma, using an ELISA with an appropriate antigen coated in a 96 well microtiter plate, indicated a good antibody response after about two months. Five, four and three days prior to cell fusion, the mice were injected intraperitoneally with approximately 50 µg of the same immunogen (no adjuvant) each time. On cell fusion, the spleen cells were extracted and fused with the myeloma cell line SP2/-Ag14 using 45% polyethylene glycol 1540 and 7% DMSO (dimethyl sulphoxide) under standard conditions (see Borrebäck, C.A.K., Eylar, M.E., *J. Biol. Chem.*, **256**:4723-4725 (1981)). Fused cells in DMEM medium supplemented with HAT (hypoxanthine, aminopterin, thymidine) were seeded into 96 well microtiter plates at a cell density of approximately 10^5 cells per well together with approximately 2 x 10^4 feeder cells per well. After about ten days, hybridoma supernatants were screened for antibody production.

Clones producing antibodies with interesting properties were identified as described below. The hybridomas were subcloned twice by limiting dilution using 96 well microtiter plates (0,5 to 1 cell per well) using mouse peritoneal macrophages as feeder cells. Stable clones producing monoclonal antibodies of interest were grown to high cell density and injected intraperitoneally into pristane-primed (0,2 ml per mouse) mice for antibody production. Antibodies were also produced on preparative scale by tissue culture (Technomouse®, Integra Biosciences). The antibody was purified from the ascitic fluid/tissue culture medium using chromatography on Protein A Sepharose® according to the instructions of the manufacturer. The cell lines were preserved frozen in 95% calf serum and 5% DMSO in liquid nitrogen.

Procedures for the identification of antibody producing clones that were specific for the complexed/-

cleaved form of PCI were carefully tailored. Microtiter plates were coated overnight with rabbit antimouse immunoglobulins (DAKO; 1 µg/well in 50 µl) in 0,1 M carbonate buffer with pH 9,6. After washing, the wells were "blocked" for 15 min with a solution of bovine serum albumin (10 mg/ml, 100 µl per well) in 50 mM Tris-HCl, 0,1 M NaCl with pH 7,4. After washing, culture medium was added to identify antibody producing clones. After washing again, the wells were incubated with a tracer agent in the above buffer for 1 h. As ^{125}I-labelled tracer agents, native PCI as well as APC:PCI complexes were used. The clones were also tested with the same method and found not to react with APC. After 1 h of incubation with the tracer agent, the plates were washed with the buffer, after which the radioactivity was measured. By having APC in the complex labelled with ^{125}I, it was possible to select antibody producing clones that bound PCI in complex with APC. Moreover, a comparison with the binding of labelled, native PCI helped to identify clones that bound PCI in complex with APC with high specific affinity, but had a low specific affinity for native PCI.

One of the antibodies (M36) with specific affinity for APC:PCI complexes and cleaved PCI was carefully characterised. Three other monoclonal antibodies were also used; one against protein C that has been characterised (Ohlin, A.K. and Stenflo, J., *vide supra*), and two against PCI which do not discriminate between cleaved and native PCI. One of the latter two antibodies has been described before (M11-5; Laurell, M., Christensson, A., Abrahamsson, P-A., Stenflo, J., Lilja, H., J. Clin. Invest., **89**:1094-1101 (1992)), whereas the other one (M52) was new (preparation disclosed herein).

Characterisation of monoclonal antibodies:

Several monoclonal antibodies with the appropriate properties were produced. Of these, M36 was carefully characterised. This monoclonal antibody was immobilised on an Affi Gel® 10 column (Biorad) and APC-complexed (A),

cleaved (B) and native (C) PCI were chromatographed on the column, as is shown in Fig. 1. The antibody bound to both cleaved and complexed PCI with high affinity, but was found not to bind or even retard the native PCI.

The dissociation constants of the antibody for its binding to PCI in complex with APC, cleaved PCI and native PCI, respectively, were measured with standard plasmon resonance technique. The dissociation constant, Kd, of M36 was 4×10^{-10} M for PCI in complex with APC and 2×10^{-10} M for the cleaved PCI, respectively, as is shown in Fig. 2. The value of Kd for the native PCI was too low, *i.e.* $\geq 10^{-5}$ M, to be determined accurately (not shown in Fig. 2). In summary, these results established that the selected monoclonal antibody M36 binds complexed PCI and cleaved PCI with very high affinity, but has very low, if any, affinity for native PCI.

As a tracer agent, a monoclonal antibody against protein C was used. This antibody, which was made by fusion of spleen cells with the myeloma cell line NS 1, is described by Ohlin, A.K. and Stenflo, J. (*vide supra*). Its epitope is in the calcium-binding N-terminal EGF-like domain of protein C, a domain exposed on both protein C and APC. Furthermore, this antibody binds to protein C and APC with high affinity, but the dissociation constant has not been quantitatively measured. The antibody binds to said epitope in a calcium-dependent manner, and it should be stated that no special requirements are made on this antibody, except that it should bind complexed APC with high affinity and can be labelled without any loss of affinity for the antigen. Among the protein C antibodies tested, this one worked best.

Collection of blood:

Blood was collected in 5 ml siliconised glass vacuum tubes (Becton Dickinson) containing 0,5 ml of a 0,129 M trisodium citrate solution, to which benzamidine had been added to a concentration of 50 mM. Usually, the samples were centrifuged within 4 h after the blood was collec-

ted, and the plasma was either assayed immediately or frozen at -70°C. The benzamidine greatly reduces the rate of complex formation between PCI and proteinases, such as kallikreine.

Performance of the assay:

The catching antibody (M36) was biotinylated with a N-hydroxysuccinimide derivative of biotin (NHS-LC-Biotin®; Pierce) according to the instructions of the manufacturer. The biotinylated protein was dialysed against a 0,1 M sodium fosfate buffer (pH 7,0) containing 0,1% NaN_3, and it was typically stored at a concentration of 0,89 mg/ml at +4°C.

To avoid the use of radioactivity and the need for enzyme conjugation of the tracer agent, *i.e.* HPC-4, a so-called DELPHIA® method was used. In said method, an Eu^{3+} labelled tracer agent is utilised, and in this assay, the DELPHIA® Eu-labelling kit 1244-302 (Wallac) was used. The HPC-4 antibody can be heavily labelled with up to 80 Eu^{3+} ions per antibody molecule without any loss of affinity for protein C. The tracer agent was stored as frozen aliquots at -20°C at a concentration of 0,043 mg/ml. Even if the DELPHIA® procedure was used in the present investigation, any commonly used means of labelling the tracer agent can probably be used.

The biotinylated M36 antibody was diluted with assay buffer (Wallac assay buffer: 50 mM Tris-HCl, 0,9% NaCl, 0,05% NaN_3, 0,01% Tween 40, 0,05% bovine immunoglobulin, 0,5 µM DPTA and 20 µg/ml cherry red) to a concentration of 2 µg/ml prior to use. The samples were analysed in duplicate. 50 µl of buffer blank, standard samples, control samples, plasma blanks and plasma (patient) samples, respectively, were added to the wells of a 96 well microtiter plate (Sero-Wel, Bibby Sterlin Ltd.) followed by addition of 50 µl of the biotinylated M36 antibody to the standard, control and plasma samples, whereas 50 µl of assay buffer was added to the other samples. The plates were then put on a LKB, Wallac shaking device for 30 s

followed by incubation at room temperature for 80 min. The entire sample was then transferred to a streptavidine coated plate (Microtitration strips, DELPHIA®, Wallac), after which the plate was shaken for 60 min on the shaking device, followed by washing in a plate washer (LKB, Wallac). 100 µl of the tracer agent solution was then added (20 ng/100 µl per well, diluted in assay buffer), followed by incubation on the shaking device for another 40 min at room temperature. After washing (Wallac wash fluid; Tris buffer, salt and Tween 20, pH 7,8), 200 µl of an enhancer solution (Wallac enhancement solution) was added to each well, and the plate was shaken on the shaking device for 10 min. Fluorescence was determined in an LKB-Wallac fluorimeter, and the APC:PCI complex formation was calculated by use of the program MultiCalc (Wallac). Here, it should be emphasized that the method according to the present invention gives a linear dose-response curve, as expected for a very high affinity antibody in a sandwich-type assay (figure 3).

In an alternative to the above procedure, the samples can be assayed directly on the streptavidine coated plate together with a biotinylated antibody, thereby avoiding the first incubation in a 96 well microtiter plate. In this manner, an even quicker procedure could be achieved without loss of performance. The time for the incubation with tracer can also be reduced. Such an alteration does not lead to any loss in the performance of the assay.

It should be noted that the standard curves are identical when made in buffer and in plasma, thus corroborating that the uncleaved inhibitor does not influence the assay. The signal given by APC:PCI complexes prior to the addition of standard has been subtracted (Fig. 3).

CLAIMS

1. A monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, said monoclonal antibody having specific affinity for both

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said inhibitor,

while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or a derivative thereof having the same biological activity.

2. A monoclonal antibody according to claim 1, wherein said monoclonal antibody is obtainable by immunisation of an animal with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved and uncomplexed form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody.

3. A monoclonal antibody according to claim 2, wherein said animal is a mouse, preferably a Balb/c mouse.

4. A monoclonal antibody according to any one of the preceding claims, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

5. A monoclonal antibody according to any one of the preceding claims, wherein said inhibitor is protein C inhibitor (PCI) or α_1-antitrypsin.

6. A method for preparation of a monoclonal antibody as defined in any one of claims 1-5, wherein an animal is immunised with a mixture of

i) a complex between a serine proteinase and an inhibitor thereof, and

ii) a cleaved form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody.

7. A method for preparation of a monoclonal antibody according to claim 6, wherein said animal is a mouse, preferably a Balb/c mouse.

8. A method for monitoring the activity of systems involving protein C inhibitor, wherein a monoclonal antibody as defined in any one of claims 1-5 is used in an immunoassay.

9. A method according to claim 8, wherein said immunoassay comprises a sandwich-type immunoassay.

10. A method according to claim 9, wherein said sandwich-type immunoassay is a technique comprising a tracer agent and said monoclonal antibody bound to a surface.

11. A method according to claim 10, wherein said tracer agent comprises an antibody having specific affinity for said serine proteinase or an epitope shared by said serine proteinase and said inhibitor.

12. A method according to claim 11, wherein said tracer agent is conjugated to a suitable enzyme and/or labelled with a tracing substance.

13. A method according to claim 12, wherein said enzyme is an alkaline phosphatase, horse radish peroxidase or a β-galactosidase.

14. A method according to claim 13, wherein said tracing substance is ^{125}I, ^{131}I, Eu^{3+} or Sm^{3+} or a similar lanthanide.

15. A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders

involving lupus anticoagulants, wherein a monoclonal antibody according to any one of claims 1-5 is utilised.

16. A method for diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to any one of claims 8-14 is utilised.

17. Use of a monoclonal antibody according to any one of claims 1-5 for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

18. Use of a method according to any one of claims 8-14 for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

19. A kit for qualitative or quantitative determination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody according to any one of claims 1-5.

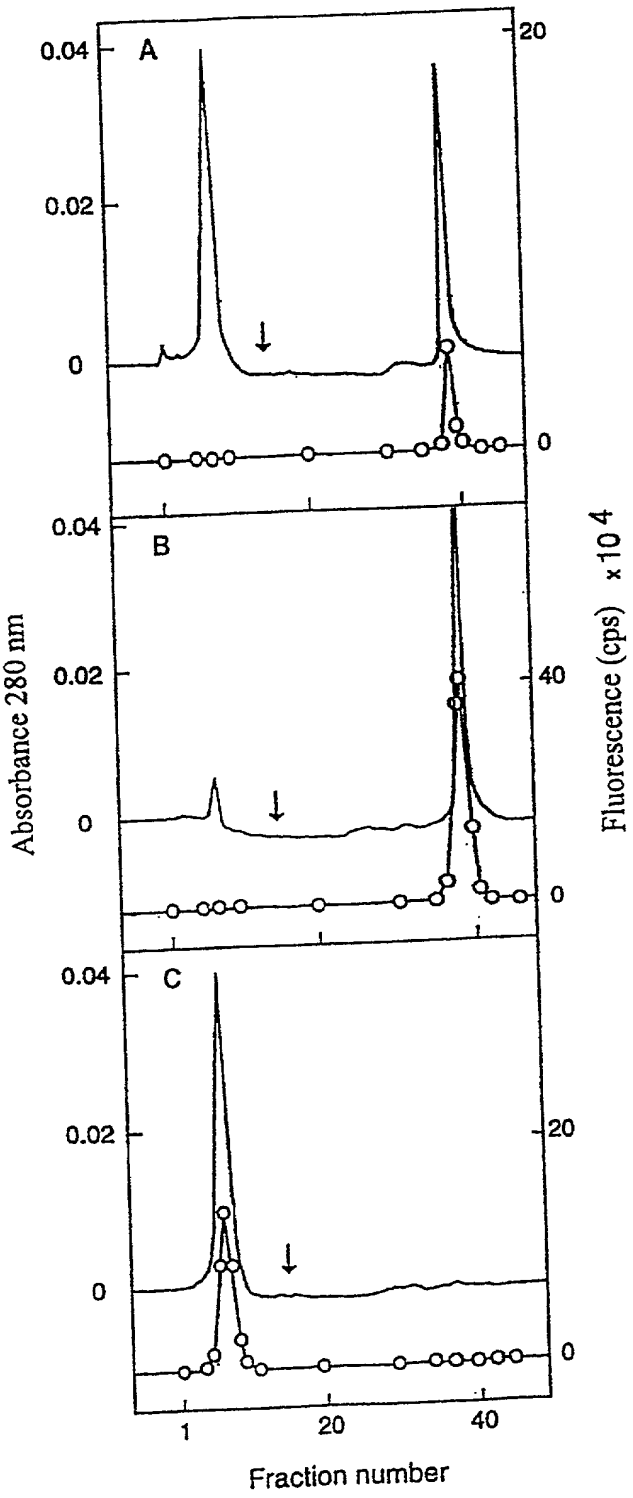
A
B
C
0.04
0.02
0
20
40
Absorbance 280 nm
Fluorescence (cps) x10 4
1
20
40
Fraction number

Figure 1

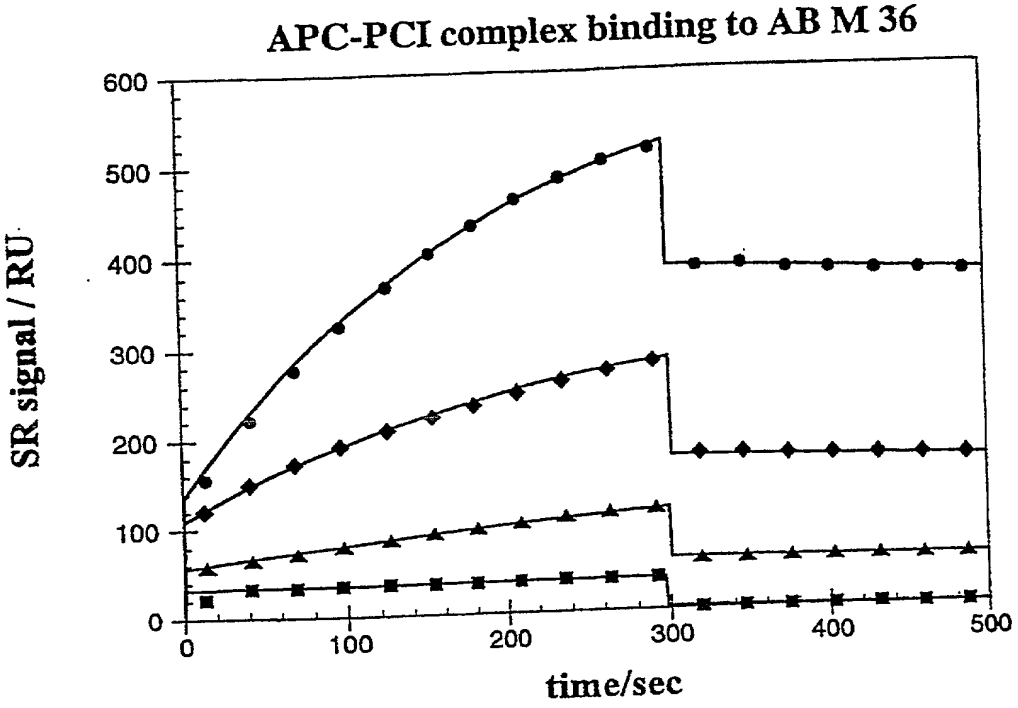
APC-PCI complex binding to AB M 36
SR signal / RU
600
500
400
300
200
100
0
0
100
200
300
400
500
time/sec

Figure 2A

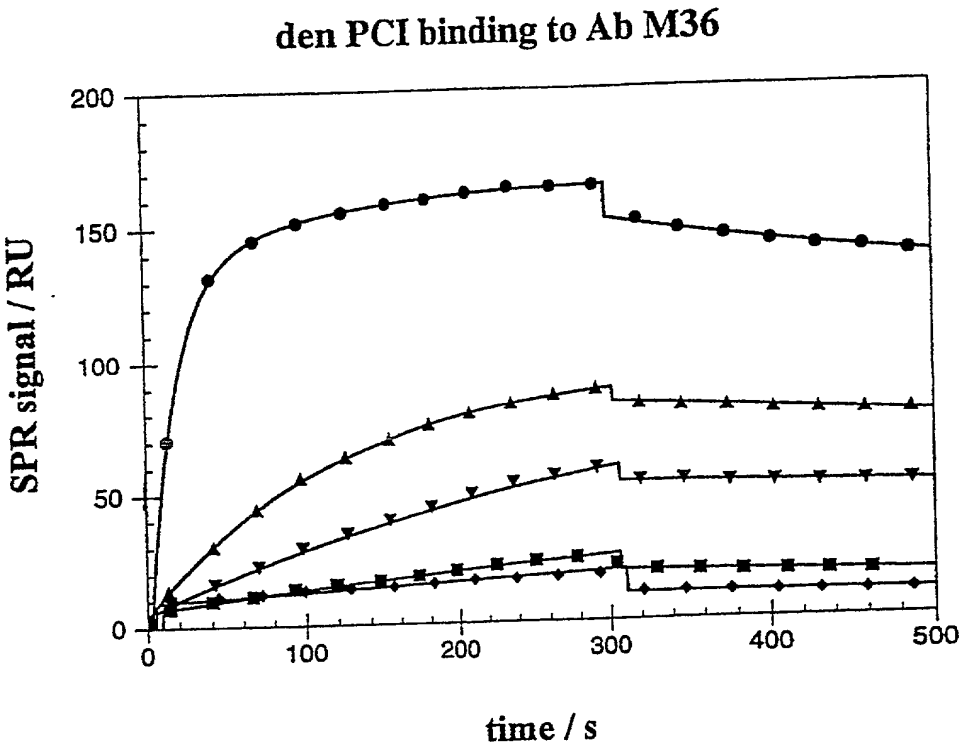
den PCI binding to Ab M36
SPR signal / RU
200
150
100
50
0
0
100
200
300
400
500
time / s

Figure 2B

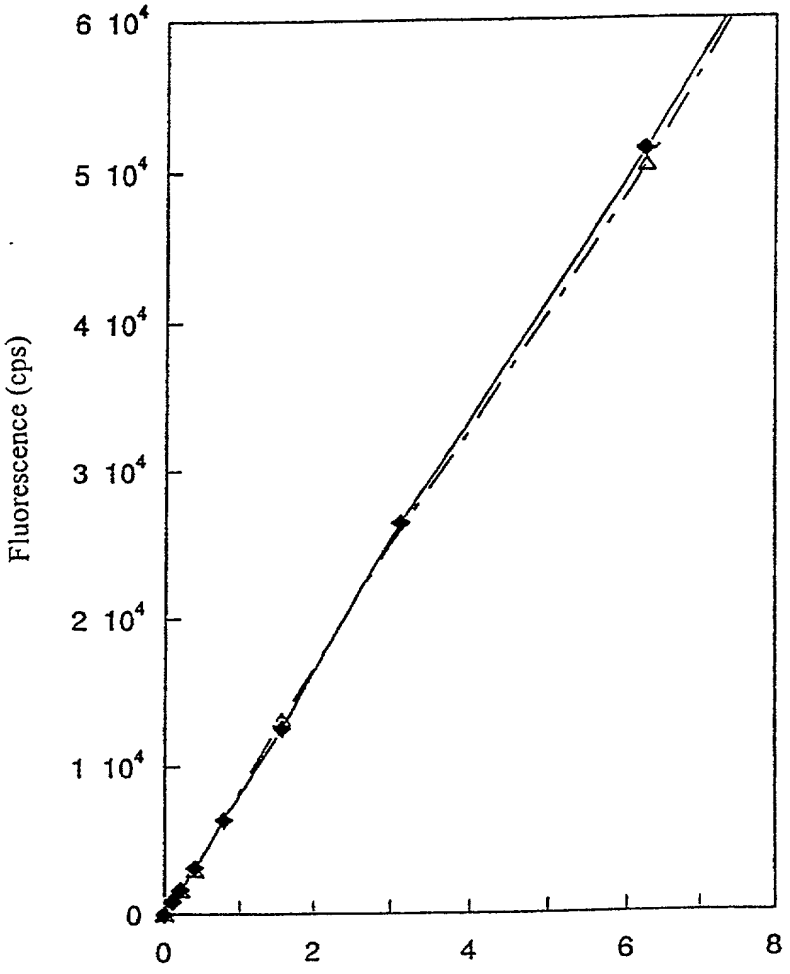
6 10⁴
5 10⁴
4 10⁴
3 10⁴
2 10⁴
1 10⁴
0
Fluorescence (cps)
0
2
4
6
8
APC-PCI-complexes (µg/L)

Figure 3

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 003300-816

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name;
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MONOCLONAL ANTIBODY

the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application

Number _____

on 8 August 2001

and was amended

on _____ (if applicable).

☐ was filed as PCT international application

Number _____

on _____

and was amended

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(e) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. §119
Sweden	9900431-9	9 February 1999	X Yes _No
			_Yes _No
			_Yes _No
			_Yes _No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

_____ (Application Number) _____ (Filing Date)

_____ (Application Number) _____ (Filing Date)

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONTINUED) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 003300-816

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to the patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS *(check one)*		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NUMBERS ASSIGNED (if any)			
SE00/00210	3 February 2000				

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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21839

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONTINUED) (Includes Reference to Provisional and PCT International Applications)	ATTORNEY'S DOCKET NO. 003300-816

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RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF THIRD JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF EIGHTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

FULL NAME OF NINTH JOINT INVENTOR, IF ANY	SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

Patent

Attorney's Docket No. 003300-816

Applicant or Patentee: Johan Stenflow

Application or Patent No.: 09/890,949

Filed or Issued: August 8, 2001

For: MONOCLONAL ANTIBODY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[X] the owner of the small business concern identified below:
[ ] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN PROTEASE AB

ADDRESS OF CONCERN c/o Stenflo, Ärtholmsvägen 196, 216 20 MALMÖ, SWEDEN

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.12, and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average, over the previous fiscal year of the concern, of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled MONOCLONAL ANTIBODY by inventor(s) Johan STENFLO described in

[ ] the specification filed herewith
[X] Application No. PCT/SE00/00210, filed 3 February 2000
[ ] Patent No. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern, or organization having rights to the invention is listed below,* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27.)

Application Serial No. 09/890,949
Attorney's Docket No. 003300-816

NAME ______________________________

ADDRESS ______________________________

[ ] individual [ ] small business concern [ ] nonprofit organization

NAME ______________________________

ADDRESS ______________________________

[ ] individual [ ] small business concern [ ] nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee and any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b).)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Johan Stenflo

TITLE OF PERSON OTHER THAN OWNER ______________________________

ADDRESS OF PERSON SIGNING Ärtholmsvägen 186
S-21620 Malmö, Sweden

SIGNATURE ______________ DATE 30 August 2001

(1/96)